

# Antiproliferative and Apoptosis-Inducing Activities of Thymoquinone in Lymphoblastic Leukemia Cell Line

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**Abstract** Acute lymphoblastic leukemia is one of the malignant proliferations of lymphoid cells in the early stages of differentiation and accounts for about 80% of all cases of childhood leukemia. Side effects of available treatment are still main concern. Thymoquinone (TQ), a natural compound isolated from *Nigella sativa*, induces growth inhibition and apoptosis in several cancer cell lines. The aim of the present study was to investigate the effect of TQ alone and in combination with doxorubicine on the proliferation inhibition and apoptosis induction of TQ in a lymphoblastic leukemia cell line. Jurkat cell line was cultured in standard condition and with concentrations of TQ (0–30  $\mu\text{M}$ ) and doxorubicine for 24, 48 and 72 h. Cell viability was measured by MTS assay. Apoptosis induction by TQ was assessed by annexin V-FITC/PI and flow cytometry analysis. TQ and DOX decreased cell viability with a time and dose dependent manner. The IC<sub>50</sub> values were  $19.461 \pm 1.141$ ,  $17.342 \pm 1.949$  and  $14.123 \pm 1.874$   $\mu\text{M}$  in 24, 48 and 72 h, respectively for TQ. IC<sub>50</sub> values for DOX were  $0.075 \pm 0.0124$ ,  $0.028 \pm 0.007$  and  $0.007 \pm 0.001$   $\mu\text{M}$  in 24, 48 and 72 h, respectively. The level of cell apoptosis in all used concentrations of TQ (4, 8, 12, 16 and 20  $\mu\text{M}$ ) was higher than control group (10.2,

14.1, 36.6, 87.5 and 93.3% respectively after 24 h; 10.7, 13.9, 64.6, 92.2 and 93.1 respectively after 48 h; 2.83, 5.83, 41.4, 71.6 and 86.6% respectively after 72 h) and reached to a significant level at 12, 16 and 20  $\mu\text{M}$  concentration for 24 and 48 h and 16 and 20  $\mu\text{M}$  for 72 h incubation. Combination of doxorubicine and TQ lead to a synergistic cytotoxicity as compared to any of them alone. The study indicated that TQ is effective on proliferation inhibition and is a strong apoptotic inducer in Jurkat lymphoblastic cell line and has synergistic effect in combination with DOX. This combination strategy can be an alternative way for more powerful anticancer effects. Therefore, the study of the mechanism of apoptosis induction of TQ can be a step forward to in target therapy which might be considered in the future studies.

**Keywords** Thymoquinone · Lymphoblastic leukemia · Apoptosis

## Introduction

Leukemias are heterogeneous malignancies [1], comprise 8% of cancers. About 80% of children leukemia cases are acute lymphoblastic leukemia [2]. Acute lymphoblastic leukemia is a malignant expansion of lymphoid precursor cells within the bone marrow, blood and extra medullary location [3], influences both children and adolescence, with a peak between the ages of 2 and 5 years and again after age of 50 [4]. Patients with T cell acute lymphoblastic leukemia (T-ALL) experience a higher recurrence and early relapse [5]. Intensification the treatment to promote survival prompts the patients to more unfavorable effects [6]. This fact derived us to new therapeutic target for the disease [5]. Over the recent years, there has been

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developing interest for naturally phytochemical components. Studies showed a regimen rich in phytochemical mixes find in plant and natural products (such as vegetables, blooms, entire grains, herbs, nuts, and seeds) are linked with cancer prevention and treatment. More than 25% of medications utilized during the last 20 years are straightforwardly gotten from plants, while the other 25% are artificially modified natural products [7]. Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone), is a phytochemical compound derived from the plant *Nigella sativa* or black cumin, which is utilized widely by Middle and Far Eastern nations [8, 9]. Different studies have exhibited that thymoquinone (TQ) has several therapeutic effects including: anti-bacterial, anti-parasitic, anti-viral, anti-inflammatory, immunomodulatory and anti-tumor properties [8, 9] and acts as an intense cytotoxic drug over a variety of human cancer cells [10] such as human breast and ovarian adenocarcinoma [11] squamous carcinoma [12], fibrosarcoma [12], laryngeal neoplastic cells [13], prostate and pancreatic cancer (PC) cell lines [14–17]. It has been recommended that TQ, as a DNA damaging factor, is a possible reactive oxygen species (ROS) producer which applies its anti-cancer properties by repressing cell growth, migration, invasion, angiogenesis and induction of apoptosis [18, 19]. The proliferation inhibitory effects of TQ is specific to cancer cells while it is less noxious to and keeps non-tumor normal cells from chemotherapy-induced damage [10, 20].

Apoptosis, also named as endogenous programmed cell death, is a basic pathway for controlling homeostasis and morphogenesis and involves in pathogens of cancers [21]. Induction of apoptosis is the most important mechanism of action in anticancer agents and its defect not only leads to progression of tumors but also increases their resistance to treatment [22, 23]. Thus, promoting apoptosis in tumors is one of the best ways for anticancer treatments [24]. We have recently reported the anti-proliferative and apoptosis effects of Pterostilbene, a phytonutrients, on T-cell lymphoblastic leukemia cell (jurkat) [25], here we examined the anti-proliferative and apoptosis induction of TQ on this cell line.

## Materials and Methods

### Cell Culture and Treatment

The human T-cell leukemia line (Jurkat, E6.1) was cultured in standard condition (95% humidity, 5% CO<sub>2</sub>, 37 °C) in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, 0.1 mg/mL streptomycin (PS) and 0.3 mg/ml L-glutamine. A 200 mM solution of TQ (Sigma-Aldrich) and DOX (Sigma-Aldrich)

was prepared in 100% DMSO (DiMethylSulfOxide, Sigma-Aldrich) and appropriate working concentrations were prepared with the cell culture medium; the final concentration of DMSO was less than 0.1% in both control and treated cells.

### Cell Viability Assay

Jurkat cells were seeded in a concentration of  $10 \times 10^3$  cells per well in 96-well plates for 24 h before treatment and exposed to TQ and DOX at different concentrations (0–30 µM), (0–0.2 µM), respectively, for different periods. Cell viability was then examined by colorimetric assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS), according to manufacturer's instruction (Promega, USA). Briefly 20 µL of MTS (5 mg/mL in phosphate buffered saline) was added to each well and incubated for 3 h, in a dark place. The absorbance was measured at 490–620 nm by an Elisa reader (stat fax-2100 awareness).

### Apoptosis Assay

Cells were seeded in 6-well plates at a density of  $2 \times 10^6$  cells per well, grown for 24 h before treatment with TQ at different concentrations for 24, 48 and 72 h. The apoptosis was examined by flow cytometry (Partec system, Germany) using the Annexin V-FITC/propidium iodide (PI) apoptosis kit (BD Biosciences), according to manufacturer's instruction. Briefly cells were washed twice with cold PBS, then cells resuspended in 1 ml of  $1 \times$  binding buffer (provided with kit) at density of  $10 \times 10^5$  100 µL of cell suspension incubated with 5 µl of Annexin V-FITC and PI for 15 min at dark at room temperature, then examined by flow cytometry. Flowjo 7.6 software was used for the analysis of the data. At least 10,000 events were recorded and represented as dot plots and histograms.

### Cytotoxicity Assay for the Combination of Thymoquinone and Doxorubicine

Jurkat cells were seeded in 96 well plate at a density of  $10 \times 10^3$  for 24 h before treatment. TQ and DOX were added at specific concentrations, and cells were incubated for 24, 48 and 72 h. Cell viability assay was performed as described above. Synergistic effect was determined by combination-index methods, derived from the median effect principle of Chou and Talalay [26] using the following formula:  $CI = C_{A,50}/IC_{50,A} + C_{B,50}/IC_{50,B}$ .  $C_{A,50}$  and  $C_{B,50}$  were the concentrations of drug A and drug B used in combination to achieve 50% drug effect;  $IC_{50,A}$  and  $IC_{50,B}$  were the concentrations of individual agents to achieve the 50% drug effect. The combination-index (CI)

method is a mathematical and quantitative representation of a two-drug pharmacologic interaction. A CI of 1 indicated an additive effect between the two agents, whereas a  $CI < 1$  or  $CI > 1$  indicated, synergism or antagonism effect, respectively.

### Statistical Analysis

Data were analyzed by SPSS using Kruskal–Wallis test and are presented as the mean  $\pm$  standard deviation from at least three independent experiments. IC<sub>50</sub> value was calculated using probit analysis.

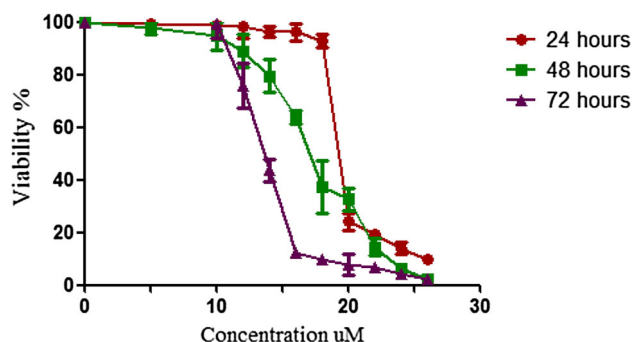
## Results

### Reduction of Cell Viability by TQ

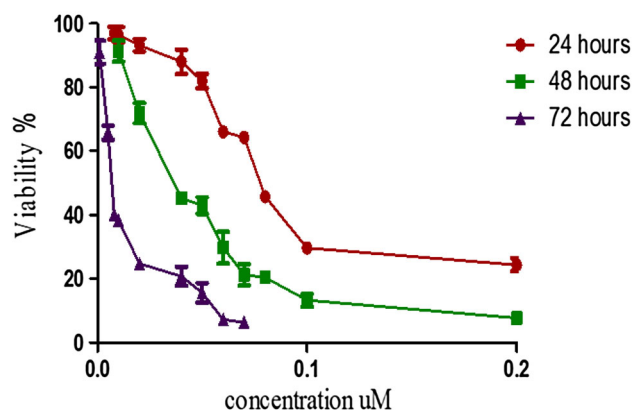
To determine the effect of TQ on viability of Jurkat cells, cells were incubated for 24, 48 and 72 h in the absence or presence of TQ at different concentrations (0–30  $\mu$ M). The ODs obtained from the absorption at 490 nm was converted to a percentage. Figure 1 shows the reduction of cell viability as concentration increased (dose-dependent). A significant reduction of Jurkat cell viability was also observed with a time-dependent manner at a specific concentration. IC<sub>50</sub> (inhibition concentration) was  $19.461 \pm 1.141$ ,  $17.342 \pm 1.949$  and  $14.123 \pm 1.874$   $\mu$ M in 24, 48 and 72 h, respectively.

### Reduction of Cell Viability by DOX

Jurkat cells, cells were incubated for 24, 48 and 72 h in the absence or presence of DOX at different concentrations (0–.2  $\mu$ M). Figure 2 shows the reduction of cell viability as concentration increased (dose-dependent). The reduction of viability was also time dependent. IC<sub>50</sub> values were



**Fig. 1** Effect of TQ on Jurkat cell viability by MTS assay. Cells were incubated with different concentrations of TQ for 24, 48 and 72 h and cell viability was determined compared to untreated cells. Reduction of cell viability was time and concentration dependent



**Fig. 2** Effect of DOX on Jurkat cell viability by MTS assay. Cells were cultured in the presence of different concentrations of DOX for 24, 48 and 72 h and viability was determined compared to untreated cells. Reduction of cell viability was time and dose dependent

$0.075 \pm .0124$ ,  $.028 \pm .007$  and  $.007 \pm .001$   $\mu$ M in 24, 48 and 72 h, respectively.

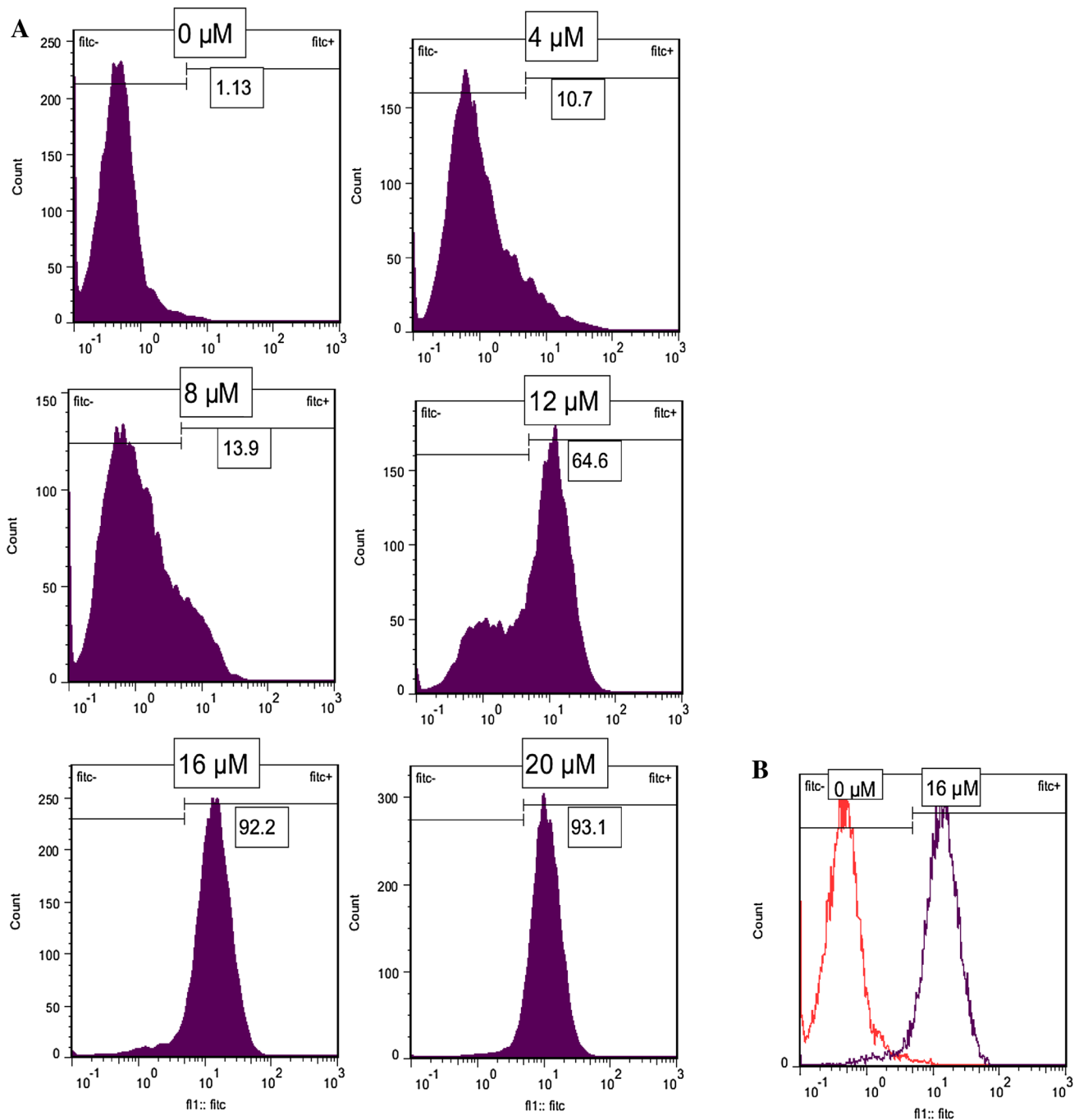
### Induction of Apoptosis by TQ

To assess whether TQ could induce apoptosis in Jurkat cells, apoptosis induction was determined by annexin-V/PI apoptosis detection kit. Cells were incubated for 24, 48 and 72 h at various concentrations (4, 8, 12, 16, and 20  $\mu$ M) of TQ. Cells that were positive just for annexin-V and those were positive both for annexin V and PI considered as early and late apoptotic cells, respectively. As shown in Fig. 3, amount of apoptotic cells are enhanced with increasing the concentration of TQ. After 48 h treatment with above concentrations, about 5.6, 2.15, 6.4, 6.45 and 3.3% of cells were early apoptotic cells and 4.81, 11.5, 57.9, 85.6 and 89.6% of cells were late apoptotic cells, Fig. 4, shows the continuous process of apoptosis. The other two time groups (24 and 72 h) showed this continuous process (data not illustrated). These data showed a significant increase in the number of apoptotic cells post treatment ( $P < 0.05$  considered significant) (Fig. 5).

As shown in Fig. 5, TQ lead to increase amount of apoptotic cell and the difference are significant at 3 concentrations (12, 16 and 20  $\mu$ M) for 24 and 48 h and 2 concentrations (16 and 20  $\mu$ M) for 72 h, compared to control. Also in each group, different concentrations were compared to each other, and were significant at 4 and 16 ( $P < 0.05$ ), 4 and 20 ( $P < 0.01$ ), 8 and 20  $\mu$ M ( $P < 0.05$ ) for 24, 48 and 72 h, respectively.

### Synergistic Effect of TQ and DOX

Cell viability was evaluated in the presence of different combinations of TQ and DOX. Stronger cytotoxicity was



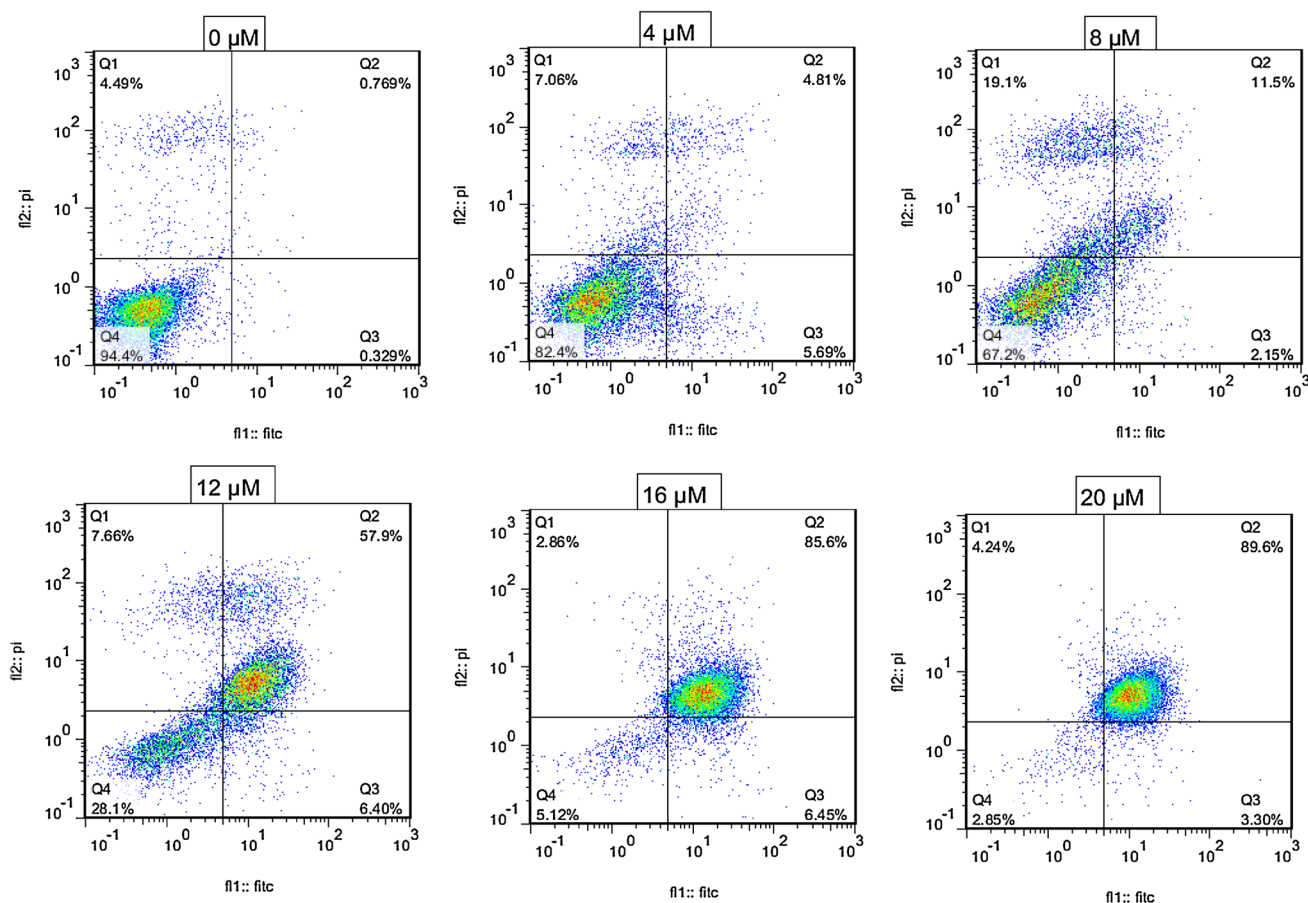
**Fig. 3** Concentration-dependent apoptosis induction by TQ in Jurkat cells. Cells were exposed to TQ at the indicated concentrations and incubated for 48 h. Apoptosis induction was assessed by flow cytometry after annexin V-FITC/PI staining. **a** Each histogram the

levels of FITC fluorescence in each histogram. **b** Fluorescence curves obtained for untreated and TQ-treated (16 μM) cells in one chart. The data are representative of three independent experiments

observed at all used combinations compared to each drug alone (Fig. 6). The results showed synergistic toxicity at 24 (data not illustrated) and 48 h. While 50% viability was observed at 0.28 μM concentration of DOX and 16 μM of TQ, a combination of 0.01 μM of DOX and 9 μM of TQ caused 50% decrease in viability (Fig. 6b). CI (combination value) were represented in Table 1.

## Discussion

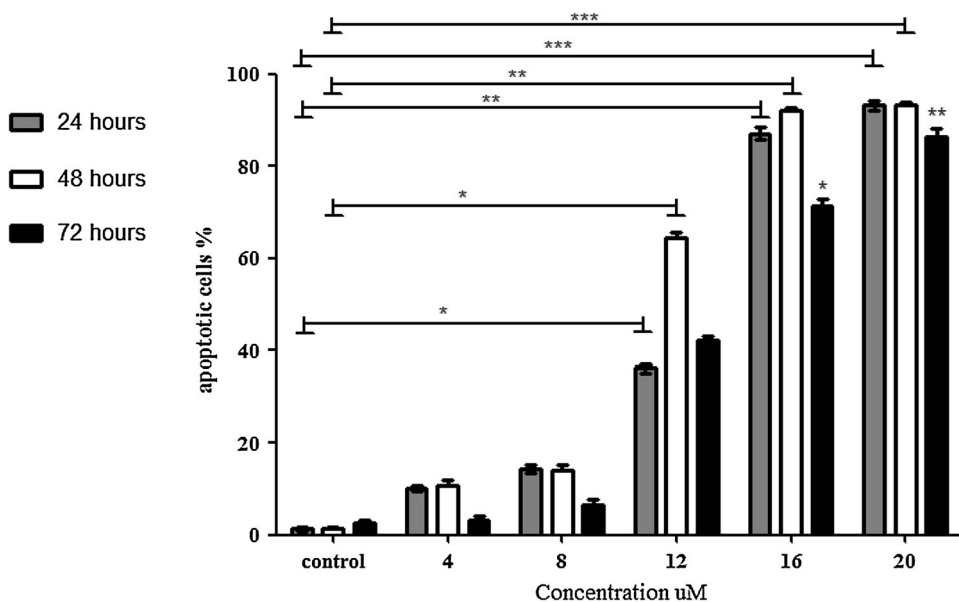
Induction of apoptosis in cancer cells is an imperative system of activity of some chemo-preventive agents [27]. Our study investigates the anti-proliferative and apoptosis induction of TQ on a lymphoblastic Leukemia cell line. TQ has already been shown to have very lower cytotoxicity on



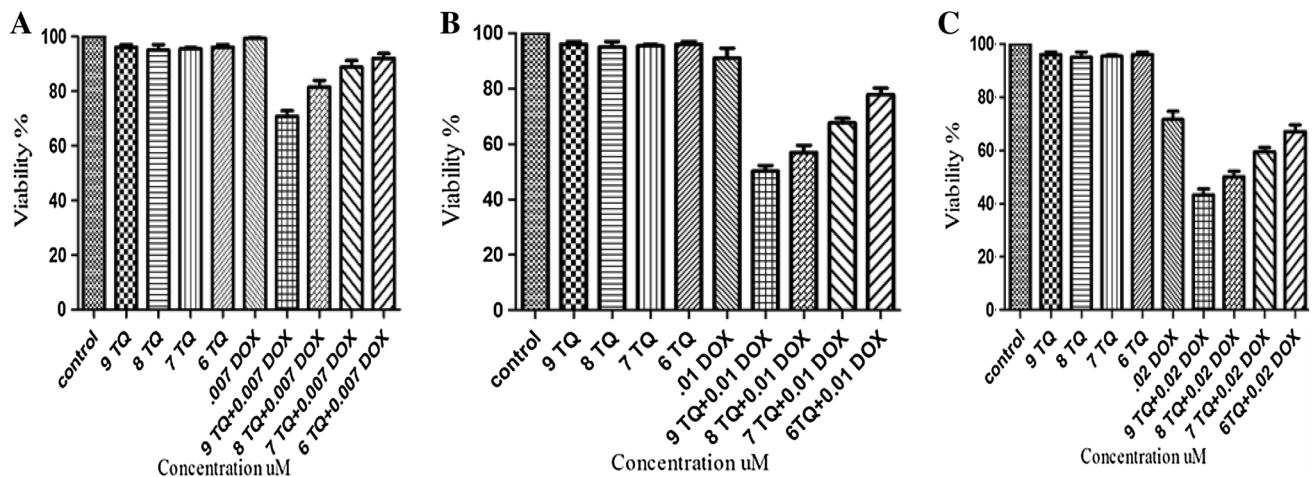
**Fig. 4** Concentration-dependent effects of TQ on the number of apoptotic cells, in Jurkat cells. Cells were exposed to different concentration of TQ (4, 8, 12, 16 and 20  $\mu$ M) for 48 h. *Dot plots* shows the results of a representative apoptosis assay. Cells of the lower left quadrant are viable (Q4); cells of the lower right quadrant

are in early apoptosis (Q3); cells of the upper left are in necrosis (Q1); cells of the upper right are in late apoptosis (Q2). The number of cells in apoptosis, expressed as percentage relative to the total cell number, is indicated

**Fig. 5** Effect of TQ on apoptosis at various concentration after 24, 48 and 72 h. Treated cells with TQ at concentration of 12, 16 and 20  $\mu$ M for 24 and 48 h and at 16 and 20  $\mu$ M for 72 h incubation, showed significant increase in the amount of apoptotic cells compared to control group (\* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001)







**Fig. 6** Jurkat cells were treated with different concentrations of TQ and DOX. A combination of DOX and TQ at 48 h, showed significant reduction in cell viability compared to each drug alone

**Table 1** Combination index values of DOX in combination with TQ on the viability of jurkat cells

Concentration of drugs	Time (h)	CI value*
10 $\mu$ M TQ + 0.03 $\mu$ M DOX	24	0.9263
9 $\mu$ M TQ + 0.01 $\mu$ M DOX	48	0.9196

\* CI = 1: additive effect, CI < 1: synergism effect, CI > 1: antagonism effect

normal PBMC than cancer cells [28, 29]. The anticancer effects of TQ have been reported to be mediated through various mechanisms such as anti-proliferation, apoptosis induction, cell cycle arrest, ROS generation and anti-metastasis/anti-angiogenesis [8]. Anti-proliferative effect of TQ has been shown in various cell lines and tumors [30–33] including drug resistant cell lines [30, 34]. Abdelfadil et al. [35] showed that TQ treatment of T28 oral cancer decrease the viability in dose-dependent manner which is consistent with our findings on Jurkat cells as we had decrease of cell viability with increase of concentration. On the other hand TQ had significant cytotoxic consequences for N28 non-tumor cells only at high concentration (100  $\mu$ M), while it was cytotoxic at dose of 50  $\mu$ M for T28 cells [35]. Chern Chiuh Woo reported that TQ can suppress the growth of various breast cancer cell lines including MCF-7, MDA-MB-231 and BT-474. This study showed the dose and time dependent anti-proliferative effect of TQ, and IC<sub>50</sub> values for above cell lines were 32, 11 and 21  $\mu$ M for 48 h, respectively [34]. To show the anti-proliferative potency of TQ, some studies compared this effect with chemotherapy drugs, as IC<sub>50</sub> values against Siha cells were 14.67 and 18.73  $\mu$ M for TQ and cisplatin, respectively, as Ng et al. reported [36]. Norfazlina et al. [37] found that TQ can inhibit cell proliferation and induces apoptosis in HL-60 cells (human myeloid

leukemia), and the inhibition concentration was about 19  $\mu$ M, that is consistency with our findings. Our findings showed that TQ can suppress cell proliferation with a time-dependent manner as the IC<sub>50</sub> was 19.461, 17.342 and 14.123 for 24, 48 and 72 h respectively. While TQ has potent cytotoxic effects on cancer cells, it had a little effect on normal cells including mouse fibroblasts (L929) [12], prostate epithelial cells (BPH-1) [33], human normal intestinal cells (FHs74Int) [38] and human normal lung fibroblast cells (IMR90) [39], which is favorable for anti-cancer therapy.

Doxorubicine like other DNA synthesis inhibitor agents has very side effects that heart toxicity is the most important of them. This drug is used in treatment of wide range of cancers such as leukemia, lymphoma and solid tumors and often used in combination with other drugs for treatment of cancers in chemotherapy regimen [40]. In this study the anti-proliferative effect of DOX on a lymphoblastic Leukemia cell line was investigated. Different studies have reported the relationship between the concentration of the drug and cytotoxic effect, as a study on breast cancer cells has shown that increasing the concentration of the drug significantly increases the rate of cell death [41]. Several studies demonstrated the effect of drug concentration and treatment duration on various cancer cells including endothelial cells, myocytes, lymphoblastic leukemia cells [42, 43]. A study conducted by sakalar and et al. [44] on T cells showed that doxorubicin lead to a reduction in DNA replication by blocking the cells in G<sub>0</sub>/G<sub>1</sub> and also leads to cancer cell death by activating the receptors Fas and caspase-dependent pathway. The results of our study was in consistent with the results of other studies.

TQ-induced apoptosis can be triggered by various ways and targeting different proteins. Previous studies

showed that induction of apoptosis by TQ can be caspase cascade dependent, as in myeloblastic leukemia HL-60 [45], or independent, as in prostatic cancer cell line [46]. Our findings showed the TQ as a powerful apoptosis inducer in jurkat cells. As shown in Fig. 3, following 24 and 48 h incubation, it derived almost all the cells to apoptosis at 16 and 20  $\mu$ M concentrations. The present study demonstrated that apoptosis induced by TQ, followed a time and dose dependent manner which is consistent with our data for cell viability. The results of our study are in agreement with the other works, showed the apoptosis induction by TQ in some other cell lines such as neuroblastoma [47], human myeloblastic leukemia [31], human breast carcinoma [34, 48], human colon cancer [38], hepatic cancer [49], and osteosarcoma cell lines [50]. Hussain et al. [51] reported the apoptotic effect of TQ in several primary effusion lymphoma (PEL) in a dose-dependent manner which is again in agreement with our study showed.

In examining the effects of DOX in combination with TQ on viability of the cells, TQ effectively increases the cytotoxic effects of doxorubicin that was very significant at 24 and 48 h. A combination of two drugs at IC50, significantly induce cytotoxic effects in cells, compare with each drug alone at the same concentration. A study by Naus et al. has shown that the combination of PolyFenol has a synergistic effect with chemotherapy (gemcitabine, 5-fluorouracil and mitomycin). Such combinations can be used to improve the efficacy of chemotherapy agents in the treatment of cancer and to enhance the cytotoxic effect given dose and to minimize side effects of the chemotherapy drugs used as single compounds or can be used as a dietary supplement with chemotherapy [52]. Another study demonstrated the effect of TQ along with gemcitabine and oxaliplatin on the pancreas cells. The results showed 2 and 3 times more cytotoxic effect in combination of drugs with TQ than any of them alone [42].

The effect of the combination of TQ and DOX on T cell lymphoblastic leukemia has not been investigated and our data demonstrated the synergistic effect of the combination.

## Conclusion

The study demonstrated that TQ can induce apoptosis in this lymphoblastic leukemia cell line which is a favorable effect of anti-cancer therapy. It was also shown that TQ enhance the effects of doxorubicin on Jurkat cells. This compound can be considered in combination with common chemotherapy agents in vitro in other cell lines and ALL lymphoblasts and experimental in vivo models.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declared no competing interests.

**Ethical Approval** This article does not contain any studies with human participants performed by any of the authors.

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